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GENETIC DIVERSITY OF *CURCUMA* IN THAILAND USING EXTERNAL TRANSCRIBED SPACER (ETS) SEQUENCES

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ABSTRACT

The Curcuma is the largest genus of Zingiberaceae which has been widely used as an important medicinal plants, spices and cosmetics. Consequently, genetic diversity from 22 samples of Curcuma was analyzed using the 5′ region of the external transcribed spacer (ETS) of 18S–26S nuclear ribosomal DNA sequences. The DNA sequences varied in length from 579-583bp. Neighbor-Joining (NJ) with Kimura -2-parameter was used to reconstruct phylogenetic trees. The genetic distance among samples ranged from 0.003-0.134. Curcuma was monophyletic entities based on ETS analyses. The phylogenetic tree showed that 22 samples of Curcuma could be separated into 2 groups with high bootstrap support. The most genetic similarity was found between C. aeruginosa and Curcuma sp. 1 which is consistent with the geographical distribution. The high bootstrap support (97%) indicated that Curcuma sp. 5, Curcuma sp. 6, Curcuma sp. 7, C. amada 1, C. amada 2, C. amada 3 and C. amada 4 were closely related species. This study represented the potential of ETS as a good candidate region for phylogenetic analysis of Curcuma species.

KEYWORDS: Genetic Diversity, Curcuma & External Transcribed Spacer (ETS)

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Original Article

INTRODUCTION

Curcuma belonging to the family Zingiberaceae or Ginger family has around 120 species distributed throughout the tropical and subtropical regions of the world (Wu and Larsen, 2000). *C. longa* L. (Turmeric) being the best-known species which curcumin, a substance in turmeric. It is commonly used as traditional medicines for many countries in Asia. Several studies suggest that it might ease symptoms of osteoarthritis and rheumatoid arthritis, like pain and inflammation. Other compounds in turmeric might also be medicinal (Záveská et al., 2012). The taxonomic identification of the genus is difficult because of morphological variation at the intraspecific level. In addition the flowering season of *Curcuma* species is short and the floral morphology has higher similarity among the species, but differs in colors and inflorescence positions. Previous studies focused on identification of *Curcuma* plants, medical and chemical compound. The relationship amongst *Curcuma* species using only morphological characters remains unclear. Therefore, an understanding of genetic diversity for breeding programs and plant genetic resource conservation is needed.

Recent molecular markers are a powerful technique for investigating the genetic diversity of plants due to independent of the influence of the environment (Murty *et al*., 2013). Molecular markers reveal polymorphism that used to study genetic relationships and genetic diversity in several plants. The ribosomal RNA (rRNA) genes and the intergenic spacer (IGS) of ribosomal RNA (the non-transcribed spacer (NTS) and external transcribed spacer (ETS)) have become widely used as a good source of phylogenetic information (Dixon and Hillis, 1993). The ETS has represented a potential data in phylogenetic studies of angiosperms (Alonso, 2014; Linder et al., 2000). The

polymorphisms of ETS region of 18S-26S of nuclear DNA (nrDNA) are good tools for identified the relationship of closely related taxes in many plant groups which DNA sequence of the 5' region of the ETS has a greater amount of variation than that of the ITS. (Markos and Baldwin, 2001; Hidalgo et al., 2006). In this study, we designed ETS primers that amplified the entire ETS throughout *Curcuma* and represented the genetic diversity of *Curcuma* in Thailand.

MATERIALS AND METHODS

Plant Materials

21 samples of *Curcuma* were collected from northern and 1 sample was collected from the northeastern region of Thailand (Table 1). *Zingibermontanum* was included in the analysis as an out-group. These plants were cultivated in Department of Biology, Faculty of Science, Naresuan University and the leaf samples were used for DNA extraction.

DNA Extraction, PCR Amplification and DNA Sequencing

Total genomic DNA was extracted from the young leaves of twenty-three samples using CTAB method (Dhakshanamoorthy and Selvaraj, 2009). The External Transcribed Spacer (ETS) region was amplified by primer 18SrRNA_ETS_R (GCCATTCGCAGTTTCACAAT) and ETS_F (TTTGCAAGTCGTGTGAGTT). PCR amplification was performed with18S rRNA External Transcribed Spacer (ETS primers). Reactions were carried out in a total volume of 20 µl consisting of 100 ng of template DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.1 mMdNTPs, 200 nM primers, 1.0 unit of *Taq* polymerase and sterile water. Amplifications were made in a Perkin Elmer 9600 thermocycler with an initial denaturing step of 3 min at 94 °C, followed by 35 cycles of 30 s at 94°C, 45 s at 52°C, 1 min at 72°C and a final extension of 5 min at 72°C. PCR products were separated by electrophoresis on 1% agarose gels in TBE buffer and visualized using ethidium bromide staining. PCR products were purified with the Nucleospin® Gel and PCR cleanup kit (MACHEREY-NAGEL, Germany) and directly sequenced at Macrogen Inc. (http://www.macrogen.com).

Data Analysis

Sequences were edited and assembled using the program Gene studio (Gene studio, Inc). The assembled contigs of species were initially aligned using Clustal W (Thompson et al., 1994) multiple sequence alignment software. The data were imported to a GENEDOC 2.6 (Nicholas et al., 1997) and manually adjusted. Neighbor-joining (NJ) was constructed using MEGA 7 program (Kumar et al., 2016). Clustering of species of each tree was confirmed by a bootstrap value of 1000 replicates. A phylogenetic tree was rooted using *Zingibermontanum*as the out-group.

Table 1: Plant Materials Used in the Study

Sample	Accession No	Source
*Curcuma aromatica*Salisb. 1	18srRNA1	Phitsanulok
*Curcuma aromatica*Salisb. 2	18srRNA31	Phetchabun
*Curcuma amada*Roxb. 1	18srRNA5	Phitsanulok
*Curcuma amada*Roxb. 2	18srRNA6	Phitsanulok
*Curcuma amada*Roxb. 3	18srRNA7	Phitsanulok
*Curcuma amada*Roxb. 4	18srRNA10	Phitsanulok
Curcuma comosa Roxb.1	18srRNA4	Phitsanulok
*Curcuma ecomata*Craib	18srRNA29	Phayao
Curcuma roscoeana Wall.*	18srRNA26	Lamphun
Curcuma longa L.	18srRNA3	Phitsanulok
*Curcuma rubescens*Roxb	18srRNA9	Phitsanulok
*Curcuma rubrobracteata*Skornickova, Sabu&Prasanthk.	18srRNA42	Tak
*Curcuma aeruginosa*Roxb.	18srRNA44	Tak

Table 1: Contd.,		
Curcuma bicolor J. Mood & K. Larsen	18srRNA55	Lampang
*Curcuma sp.*1	18srRNA43	Tak
*Curcuma sp.*2	18srRNA17	Lampang
*Curcuma sp.*3	18srRNA30	Lampang
*Curcuma sp.*4	18srRNA40	Lampang
Curcuma sp 5	18srRNA47	Nakronrachasima
Curcuma sp 6	18srRNA16	Phayao
Curcuma sp 7	18srRNA41	Phitsanulok
Curcuma sp 8	18srRNA2	Phitsanulok
Zingibermontanum	18srRNA33	Phitsanulok

RESULTS AND DISCUSSIONS

Sequences Characteristics

All samples within *Curcuma* produced amplification products when PCR was performed with the ETS primers using the same protocol (Table 1). The length of ETS varied from 579bp (*C. aeruginosa*) to 583 (*Curcuma sp.* 6, *Curcuma sp.* 7, *C. amada* 1 and *C. amada* 4) and averaged 581bp. The sequence alignment contained 584 bp and 87 were parsimony informative sites. The CG content was 49.4% and AT was 50.6%. Furthermore, the average number of substitutions per site was 0.053. The sequence alignment showed the nucleotide substitution that occurred in the 3 region of 4 samples (*C. Bicolor*, *Curcuma sp.* 2, 3 and 4). They shared 40 sites of 1 base substitution site, 4 sites of 2 base substitution site and 1 site of 5 base substitution. Accordingly, to nucleotide substitutions have occurred throughout the 3 region of the ETS the *Calycadenia* and *Osmadenia* (Baldwin and Markos, 1998). Interestingly, only 1 insertion was found in some species (*C. amada* 1, 2, 3, 4 and *Curcuma sp.* 5, 6, 7) (Figure 1)

Phylogenetic Analysis

NJ tree was constructed using Kimura 2-parameter model of nucleotide substitution. The genetic distance revealed that the species fell in the range of 0.003-0.134 among 22 samples of *Curcuma* (Table 2). Maximum genetic distance was between *Curcuma sp.* 5 and *C. Bicolor* (0.132), while the least genetic distance was found between *Curcuma sp.* 1 and *C. aeruginosa* as same as *C. amada*2 and *C. amada* 3 (0.002).The high level of ETS sequences similarity has been found between intragenomic (Poczai and Hyvonen, 2010). Phylogenetic trees obtained in the ETS region showed high support values. Two major clades were identified with ≥ 99 % bootstrap support and *Zingibermontanum* as the out-group. *C. Bicolor*, *Curcuma sp.* 2, 3 and 4 were clustered into the same group (100%) and other samples were placed in the second group (99%). *Curcuma sp.* 5, 6, 7 and *C. amada* 1,2,3,4 was placed within the same sub-clade with strong bootstrap support (97%) as same as *Curcuma sp.* 1 and *C. aeruginosa* (100%) which were also in accordance with geographical origin. *C. amada*, *C. aromatic* and *C. comosa* were group in the same clade which could be correlated with chromosome number (Skornickova, et al., 2007). This result indicated that the three species were closely related. The result showed that the *Zingiber* and *Curcuma* were grouped separately agreement with the study was to examine the genetic diversity among and within two populations of Zingiberaceae (*Zingiber* and *Curcuma*) using DNA-based molecular markers such as RAPD, ISSR and SSR (Mohanty, et al., 2014). However, *C. aromatic* 1 and *C. aromatic* 2 were placed in the difference sub-clade because *C. aromatica* is a seed setting species and seedling variation may be the reason for the genetic variation in the two samples of *C. aromatica* (Syamkumar and Sasikumar, 2007)

The results of the present study showed that the ETS primers were highly effective in amplifying and sequencing

of *Curcuma* species. The ETS sequences represented that this was a useful region for phylogenetic relationships and genetic diversity studies in *Curcuma*. Most of the previous studies revealed that the ETS was more variable and phylogenetically informative than the ITS region in some plants (Linder et al., 2000; Logacheva. et al., 2010)

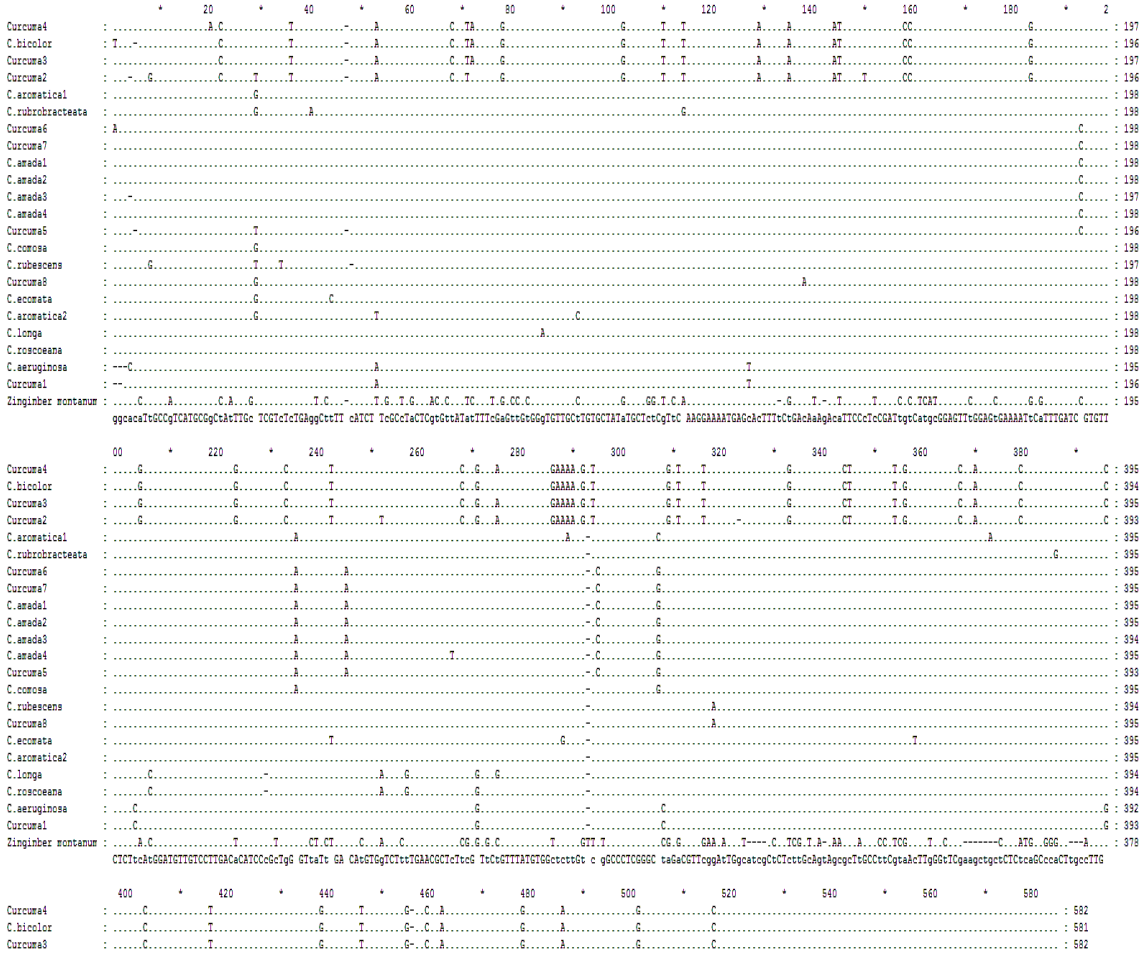

Figure 1: Alignment of ETS Region from 22 *Curcuma* Samples and *Zingibermontanum*

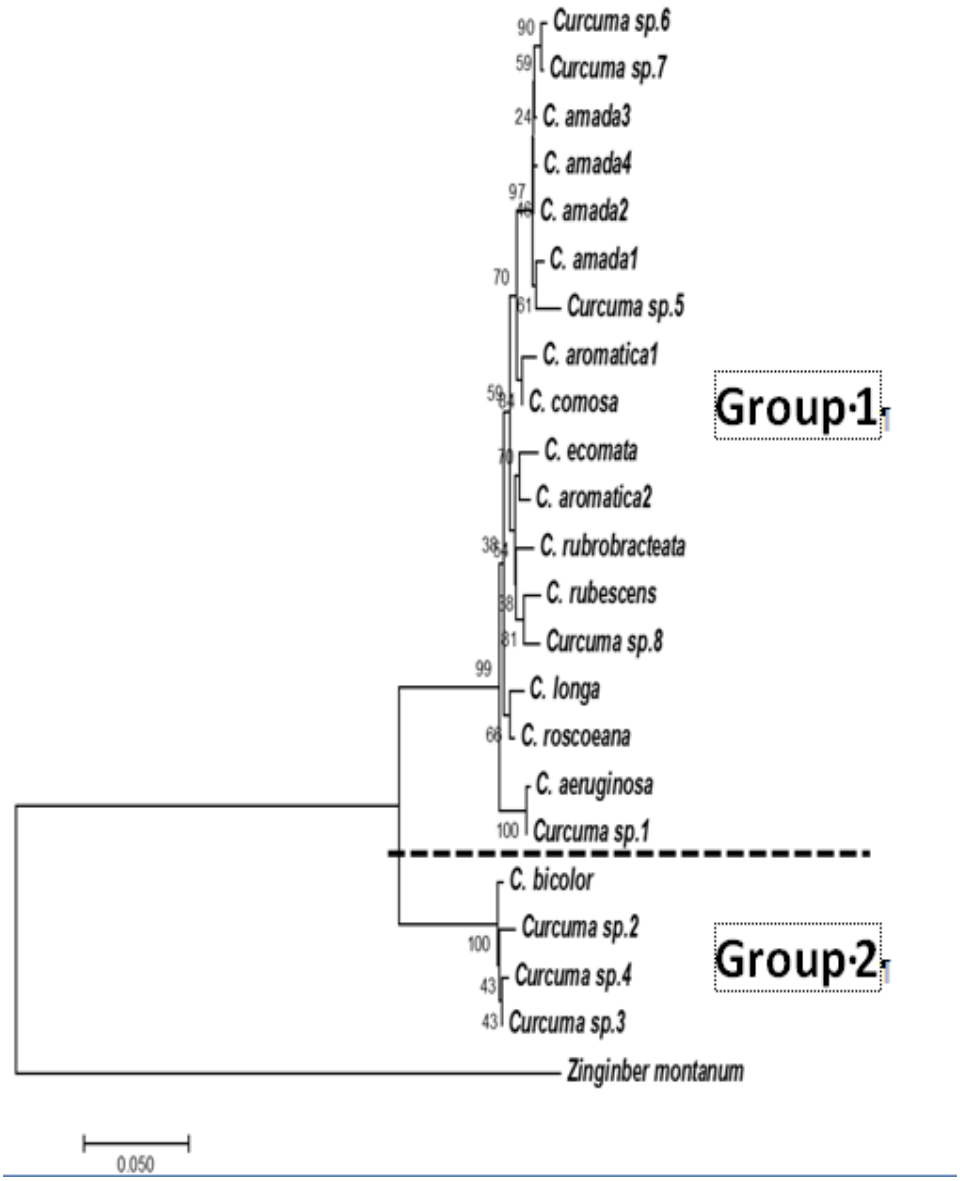

Figure 2: Dendroagram show the Relationships Among the 22 Accession of Curcuma. The Dendrogram was Generated from Similarity Index Based on NJ

Table 2: Estimate of Evolutionary Divergence between Sequences

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. Curcuma sp.4																							
2. C. bicolor	0.005																						
3. Curcuma sp.3	0.003	0.005																					
4. Curcuma sp.2	0.010	0.012	0.010																				
5. C. aromatica1	0.114	0.111	0.109	0.116																			
6. C. rubrobracteata	0.117	0.115	0.113	0.120	0.021																		
7. Curcuma sp.6	0.124	0.120	0.122	0.128	0.025	0.030																	
8. Curcuma sp.7	0.118	0.114	0.116	0.122	0.023	0.028	0.003																
9. C. amada1	0.123	0.121	0.119	0.128	0.023	0.028	0.012	0.010															
10. C. amada4	0.119	0.117	0.115	0.124	0.019	0.025	0.009	0.007	0.007														
11. Curcuma sp.5	0.134	0.132	0.130	0.134	0.030	0.035	0.019	0.019	0.016	0.016													
12. C. amada2	0.117	0.115	0.113	0.122	0.017	0.023	0.007	0.005	0.005	0.002	0.014												
13. C. amada3	0.115	0.113	0.115	0.120	0.019	0.025	0.007	0.005	0.007	0.003	0.016	0.002											
14. C. comosa	0.113	0.111	0.109	0.116	0.007	0.016	0.017	0.016	0.016	0.012	0.023	0.010	0.012										
15. C. rubescens	0.116	0.114	0.116	0.112	0.026	0.019	0.032	0.026	0.032	0.028	0.037	0.026	0.025	0.021									
16. Curcuma sp.8	0.119	0.117	0.119	0.122	0.025	0.021	0.032	0.030	0.032	0.028	0.035	0.026	0.025	0.019	0.016								
17. C. ecomata	0.116	0.114	0.112	0.118	0.023	0.019	0.032	0.030	0.030	0.026	0.037	0.025	0.026	0.017	0.025	0.023							
18. C. aromatica2	0.115	0.113	0.111	0.118	0.019	0.016	0.028	0.026	0.026	0.023	0.033	0.021	0.023	0.014	0.021	0.019	0.014						
19. C. longa	0.116	0.116	0.112	0.118	0.026	0.026	0.028	0.026	0.026	0.023	0.035	0.021	0.023	0.021	0.030	0.030	0.028	0.021					
20. C. roscoeana	0.107	0.105	0.107	0.109	0.021	0.021	0.025	0.023	0.025	0.021	0.033	0.019	0.017	0.016	0.021	0.021	0.023	0.019	0.009				
21. C. aeruginosa	0.118	0.114	0.114	0.122	0.032	0.032	0.034	0.032	0.035	0.032	0.045	0.030	0.032	0.026	0.036	0.035	0.034	0.028	0.030	0.025			
22. Curcuma sp1	0.115	0.113	0.111	0.120	0.030	0.030	0.035	0.034	0.034	0.030	0.043	0.028	0.030	0.025	0.034	0.034	0.032	0.026	0.028	0.023	0.002		
23. Z. montanum	0.497	0.490	0.493	0.505	0.519	0.497	0.515	0.516	0.518	0.514	0.516	0.511	0.511	0.511	0.512	0.511	0.511	0.497	0.496	0.489	0.504	0.503	

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